Expression of taurine transporter is regulated through the TonE (tonicity-responsive element)/TonEBP (TonE-binding protein) pathway and contributes to cytoprotection in HepG2 cells

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In hypertonic environment, taurine accumulates in cells via activation of TauT (taurine transporter) as an adaptive regulation. Recent studies revealed that TonE (tonicity-responsive element)/TonEBP (TonE-binding protein) pathway regulated the expression of various molecules which protect cells against hypertonic stress. In the present study, we investigated the osmoregulatory mechanisms of TauT expression. TauT was up-regulated at both functional and transcriptional levels in HepG2 under hypertonic condition. The TonE site was identified in the promoter region of *TauT* gene. Reporter gene assay revealed that promoter activity was increased under hypertonic conditions, whereas deletion or mutation of TonE sequence abolished the induction of the promoter activity in response to hypertonicity. By using the reporter gene plasmids containing a TonE site of TauT promoter (p2xTonE-Luc), it was demonstrated that a TonE site was sufficient for the hypertonicity-mediated activation of TauT promoter. Importantly, co-transfection of TauT promoter gene plasmid with wild-type TonEBP expression vector enhanced promoter activity under isotonic conditions, whereas dominant-negative TonEBP abrogated the TauT promoter activity induced by hypertonicity. Finally, treatment with taurine prevented HepG2 cells from cell death induced by hypertonic medium. These findings suggested that induction of TauT by hypertonicity is mediated by the activation of the TonE/TonEBP pathway and confers resistance to hypertonic stress.

Key words: osmolyte, osmoregulation, promoter assay, taurine transporter, tonicity-responsive element-binding protein (TonEBP), transcription factor.

INTRODUCTION

Taurine is one of the most abundant free amino acids in mammalian tissues. Taurine contributes to many physiological functions, such as modulation of ion movement, regulation of intracellular osmolality, conjugation with bile acids, detoxification and membrane stabilization [1–6]. Recently, taurine has been reported to prevent cell injury induced by various stimulations, such as hypoxia [7,8]. Although taurine is biosynthesized from methionine and cysteine, the tissue taurine content is determined by the uptake from plasma. Taurine is taken up into cells via a specific transport system, Na^+, Cl^--dependent TauT (taurine transporter). Intracellular taurine concentrations are often as high as 10 mmol/kg of wet tissue in many mammalian tissues, whereas taurine is found at the concentration of 20–100 μM in plasma [2,6,9], implying that TauT plays an important role in the maintenance of high concentration of taurine in tissues.

Extracellular hyperosmolality results in cell damage by disturbing the intracellular ionic homoeostasis. At the same time, hyperosmolality is supposed to be an important extracellular signal, which regulates a wide range of target genes, including amino acid transport system 2 [10], aldose reductase [11] and $Na^+/K^+/2Cl^-$ co-transporter [12], at the transcriptional level, as an adaptive response. Interestingly, TauT mRNA is increased in response to abnormal intracellular osmolality in various tissues [13] although the signalling pathway remains unclear.

Recently, the promoter region of rat *TauT* gene was identified [14]. It was then demonstrated that this promoter region contained the binding sites of p53 or WT-1, and that these transcriptional factors regulated TauT mRNA expression [15,16]. In the present study, by using promoter-reporter plasmids, we examined the molecular mechanisms of the up-regulation of TauT by hypertonicity, and we also analysed the biological significances of the up-regulation of TauT under hypertonic conditions. Our results provide new insights into the anti-hypertonic response through the taurine transport system.

METHODS

Plasmids

A DNA fragment of TauT promoter region positioning from -416 to $+66$ [14] was amplified by PCR using Wister Kyoto rat genomic DNA as a template and was cloned into pCR2.1 by using TOPO Cloning kit (Invitrogen, Carlsbad, CA, U.S.A.). Then this fragment was used as the template for the preparation of different lengths of TauT promoter region. Primer sequences are shown in Table 1. Three different lengths of TauT promoter region from positions -269 to $+46$, -124 to $+46$ and -99 to $+46$ were prepared by PCR and inserted into firefly luciferase plasmids pGL3 (Promega, Madison, WI, U.S.A.), pTauT/$-$269-Luc, pTauT/$-$124-Luc and pTauT/$-$99-Luc. Mutation of the TonE (tonicity-responsive element) site was generated in pTauT/$-$124-Luc by PCR using the forward primer $-$124m (shown in Table 1). This PCR product was inserted into pGL3 to create pTauT/$-$124mut-Luc. The plasmids were verified by sequencing. The reporter plasmid p2xTonE-Luc was generated by insertion of two copies of the double-stranded oligonucleotide containing TonE of TauT promoter region, 5′-GGCCAAGCTGGTATTTTTCCACCCAGCAGG-3′ into pGL3. The expression vector of TonEBP (TonE-binding protein), pFLAG-TonEBP, was a gift from Dr B. C. Ko (University of Hong Kong, Hong Kong,

Abbreviations used: DN, dominant negative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium, inner salt; TauT, taurine transporter; TonE, tonicity-responsive element; TonEBP, TonE-binding protein.

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Table 1 PCR primers

Primer	Sequence
Forward	
− 416	5′-CTGTGGTGTGCTAATGGCTATCA-3′
− 269	5′-GGGGTACCCGGGTTCTTTGTG-3′
− 124	5′-GGGGTACCCGGCCAAGCTGGTATT-3′
− 99	5′-GGGGTACCCAGCAGGATGGGTGAT-3′
− 124m	5′-GGGGTACCCGGCCAAGCTG**atcttccctta**CCCA-3′
Reverse	
+ 66	5′-GATCGCGGCGTTGGC-3′
+ 46	5′-AAAAGCTTCTAGATGGCACGGGAGTTCA-3′

People's Republic of China) [11]. The DN (dominant negative)-TonEBP construct was cloned into pFLAG-CMV2 (Sigma–Aldrich, St. Louis, MO, U.S.A.), pFLAG-DN-TonEBP.

Cell culture

HepG2 cells were maintained in Eagle's minimal essential medium supplemented with 10 % fetal bovine serum. After culturing in serum-free medium for 24 h, HepG2 cells were exposed to osmotic stress by culturing in hypertonic media. Hypertonic media were prepared by adding 50 or 100 mM sodium chloride at a final concentration of 166 or 216 mM (NaCl + 50 or NaCl + 100) respectively or by adding sucrose at a final concentration of 50 or 100 mM (Suc 50 or Suc 100). Previous studies have produced hypertonicity by using either permeable agents, such as sodium chloride or impermeable agents, e.g. sucrose and mannitol [11,17–20]. In the present study, to confirm that the experimental results did not depend on the permeability of the reagents, we generated two kinds of hypertonic conditions by using sodium chloride or sucrose.

Northern-blot analysis

Human TauT cDNA, a gift from Dr H. Satsu and Dr M. Shimizu (University of Tokyo, Tokyo, Japan) [21], and rat GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA were labelled with [α-^{32}P]dCTP (PerkinElmer Life Sciences, Boston, MA, U.S.A.) using MegaPrime DNA-labeling system (Amersham Biosciences, Little Chalfont, Bucks., U.K.). Total RNA of HepG2 cells was isolated by using TRIzol® (Gibco BRL, Gaithersburg, MD, U.S.A.) according to the manufacture's instructions. Total RNA (20 μg) was size-fractionated on 1.5 % agarose gel containing 2.2 M formamide and was blotted on to a nylon membrane. The membrane was hybridized by using QuickHyb hybridization solution (Stratagene, La Jolla, CA, U.S.A.) and was then washed with a buffer containing 0.2 × SSC plus 0.1 % SDS, and autoradiographed. The intensity of the bands of TauT mRNA was measured by an image analyser (Fuji Film, Tokyo, Japan) and was normalized to that of GAPDH mRNA.

Western-blot analysis

Cells were lysed directly with SDS/PAGE solution, and the lysate was immediately boiled for 5 min. The lysate was electrophoresed on SDS gel, transferred on to PVDF membrane and immunoblotted with anti-TauT polyclonal antibody (1:1000; Alpha Diagnostic International, San Antonio, TX, U.S.A.) or anti-actin monoclonal antibody (1:1000; Sigma). The ECL® system (Amersham Biosciences) was used for detection.

Taurine uptake measurement

Taurine uptake activity was measured as described previously [22]. After the culture medium was removed, cells were washed twice with uptake medium before the initiation of uptake measurements. Then cells were incubated with uptake buffer containing 5 μM [1,2-^{3}H]taurine (0.1 μCi/ml) for 10 min and were quickly washed with cold PBS and were lysed with 0.1 M NaOH. Radioactivity of the lysate was measured by liquid-scintillation spectrometry. Results were normalized with protein content.

Luciferase assay

Transient transfection into HepG2 cells was performed by the calcium phosphate method using CellPhect transfection kit (Amersham Biosciences). The *Renilla* luciferase plasmids, pRL-thymidine kinase promoter (pRL-tk), were co-transfected as a control for the transfection efficiency. The cells were then cultured in isotonic or hypertonic medium for 24 h and were harvested thereafter. The firefly and *Renilla* luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega).

Cell survival assay

Cells were plated on 96-well plates at a density of (1–5) × 10^4 cells/well and were cultured in 10 % serum-containing medium for 3 days. After culturing in serum-free medium for 24 h, cells were cultured in hypertonic medium containing 100 mM sucrose with or without taurine or β-alanine at 20 μM for 24–48 h. Cell viability was measured with the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium, inner salt] system by using CellTiter 96 AQuous One Solution Cell Proliferation Assay kit (Promega), according to the manufacturer's instructions.

Statistical analysis

Statistical significance was determined by Student's *t* test. Each value was expressed as the means ± S.D. Differences were considered statistically significant when the calculated *P* value was < 0.05.

RESULTS

Osmoregulation of taurine uptake activity at the transcriptional level in HepG2 cells

To examine whether TauT gene expression is regulated by osmolality in HepG2 cells, Northern-blot analyses were performed (Figure 1). TauT mRNA was significantly up-regulated by culturing in hypertonic media, NaCl + 50, Suc 50 or Suc 100 for 24 h (Figure 1). The amounts of total RNA and protein prepared from the cells cultured in hypertonic medium containing 216 mM NaCl (NaCl + 100) were significantly decreased compared with those from other conditions (results not shown), possibly due to the cytotoxicity of 216 mM NaCl. Thus, in the present study, hypertonic condition, NaCl + 100, was not used.

To determine the regulation of TauT protein, Western-blot analyses were performed. As shown in Figure 2, TauT protein was up-regulated by hypertonicity.

Importantly, in parallel with mRNA and protein expression, taurine uptake activity was increased in hypertonic media (Figure 3).

Identification of TonE of rat TauT gene promoter region

Recently, the rat TauT promoter region has been reported [14]. Interestingly, we identified the consensus sequence of TonE at

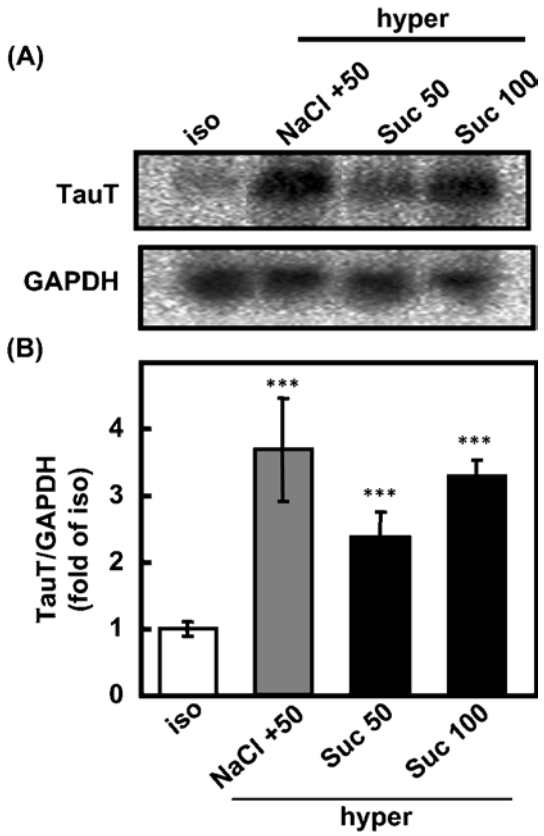

Figure 1 Osmoregulation of TauT mRNA expression in HepG2 cells

TauT mRNA was analysed by Northern blotting (**A**). HepG2 cells were cultured in isotonic (iso) or hypertonic (hyper; NaCl + 50, Suc 50 or Suc 100) media for 24 h. Results (**B**) are normalized to GAPDH mRNA expression and are means ± S.D., $n = 4$. ***$P < 0.001$ versus isotonic condition.

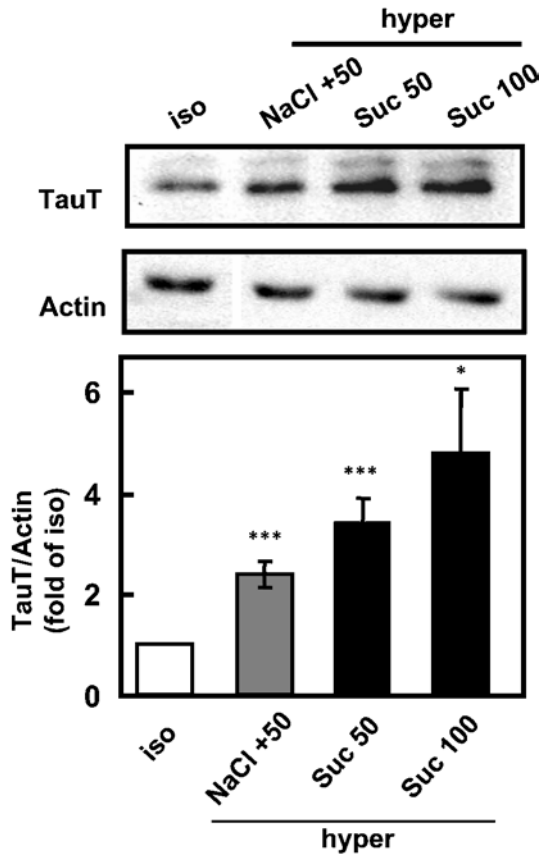

Figure 2 Osmoregulation of TauT protein expression in HepG2 cells

TauT protein was detected by Western blotting. HepG2 cells were cultured in isotonic (iso) or hypertonic (hyper; NaCl + 50, Suc 50 or Suc 100) media for 24 h. Cell lysates were immunoblotted with anti-TauT or anti-actin antibody. Results are normalized to actin expression and are means ± S.D., $n = 6$. *$P < 0.05$, ***$P < 0.001$ versus isotonic condition.

− 110 bp from the transcriptional start site (Figure 4A). To compare the nucleotide sequence alignments of TauT promoter region among mammals, the sequence homology search was performed by using human or mouse genome databases (http://www.ncbi.nlm.nih.gov/BLAST/). As a result, significant similarities were found with the upstream of mouse (96 %) and human (90 %) *TauT* gene (NCBI accession numbers NT_039353 and NT_005927). Especially, the TonE site was completely conserved (Figure 4B).

To examine whether or not promoter activity of this region was regulated by osmolality, the reporter constructs, pTauT/− 269-Luc, pTau/T − 124-Luc or pTauT − 99-Luc, were transiently transfected into HepG2 cells. Transfected cells were cultured in hypertonic media for 24 h, and then luciferase activity was measured (Figure 4C). The reporter gene plasmids, pTauT/− 269-Luc and pTauT/− 124-Luc, which contain TonE, showed an increase in the promoter activity in response to hypertonicity, but pTauT − 99-Luc, which lacks TonE, did not. Significantly, mutagenesis at the TonE site eliminated the induction of promoter activity by hypertonicity (Figure 4D). These results suggested that the TonE consensus sequence existing from − 124 to − 99 bp of TauT 5′-flanking region was essential for the osmoregulation of *TauT* gene expression.

To determine further whether the TonE consensus site is responsible for TauT promoter activity, p2xTonE-Luc containing two TonE sequences of TauT promoter region was generated.

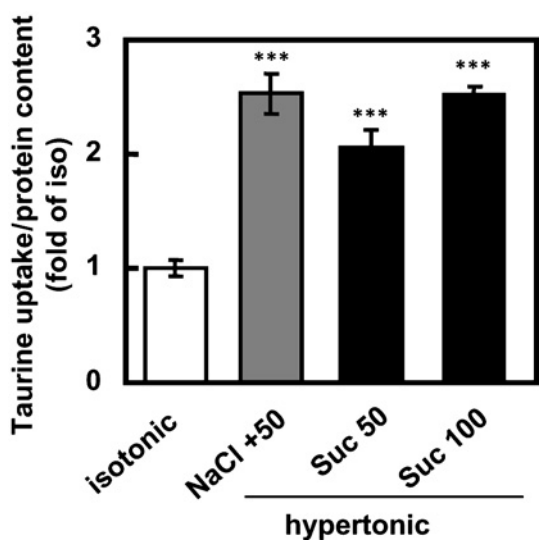

Figure 3 Osmoregulation of taurine uptake activity in HepG2 cells

Cells were cultured in isotonic or hypertonic (NaCl + 50, Suc 50 or Suc 100) media, and taurine uptake activity was measured by using radiolabelled taurine. Results are normalized to protein content and are means ± S.D., $n = 4$. ***$P < 0.001$ versus isotonic condition.

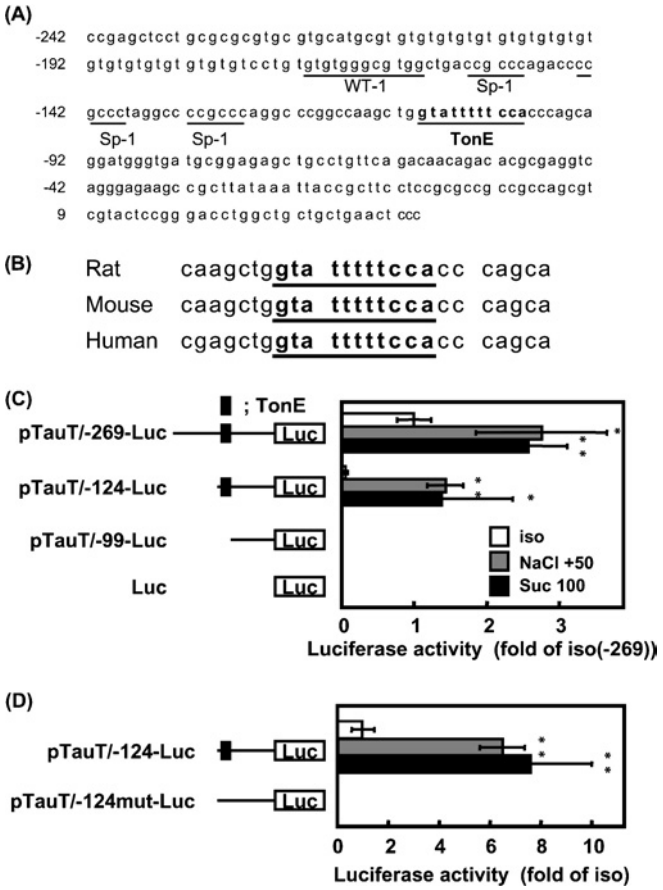

Figure 4 Osmoregulation of the promoter activity of a *TauT* gene depends on TonE

(**A**) DNA sequence of the promoter region of rat *TauT* gene. Consensus sequences of WT-1, Sp-1 and TonE are underlined. (**B**) Nucleotide sequence alignment of the 5′-flanking region of *TauT* gene among rat, mouse and human. (**C**, **D**) Cells were transfected with promoter-reporter constructs. Cells were cultured 24 h after transfection in isotonic (iso) or hypertonic media (NaCl + 50 or Suc 100) for 24 h. Promoter activity was normalized with luciferase activity of pRL-tk. Results are means ± S.D., $n = 4$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus isotonic condition.

Luciferase activity of p2xTonE-Luc was remarkably increased by exposure to hypertonic media (Figure 5A).

Co-transfection of TonEBP or DN-TonEBP

To reveal the significance of TonEBP in TauT promoter activity, the expression vectors encoding the wild-type or DN form of TonEBP were co-transfected with reporter plasmids into HepG2

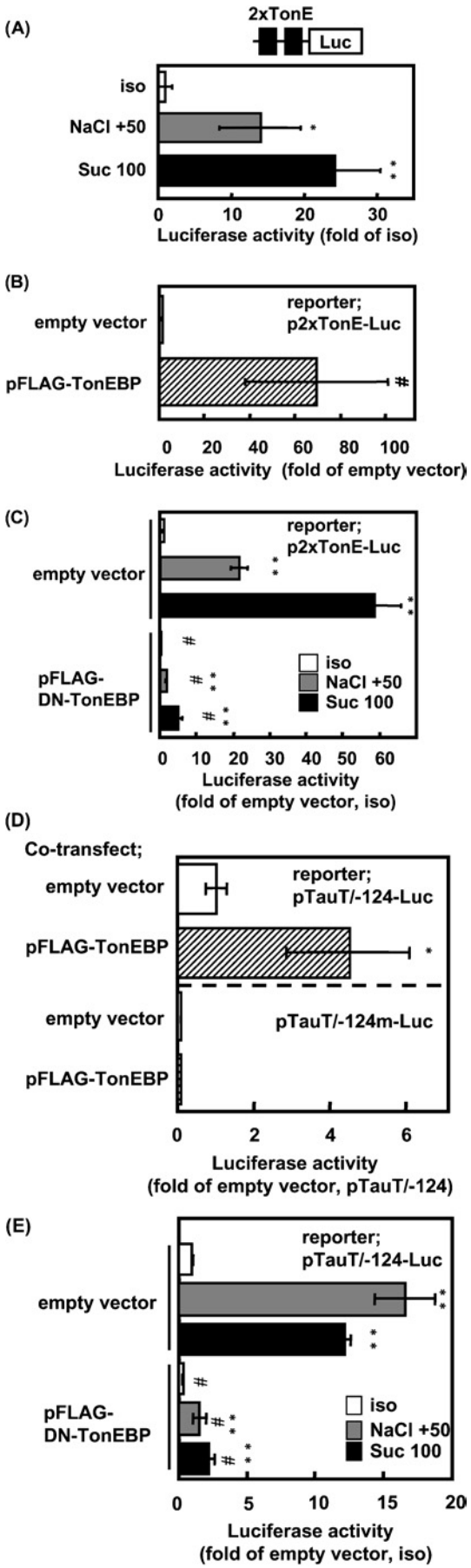

Figure 5 TauT promoter activity is regulated through a TonE/TonEBP pathway

(**A**) Cells were transfected with p2xTonE-Luc. Cells were cultured 24 h after transfection in isotonic (iso) or hypertonic media (NaCl + 50 or Suc 100) for 24 h. Results were obtained from three experiments. (**B**, **D**) A pFLAG-TonEBP or empty vector was co-transfected with p2xTonE-Luc (**B**), pTauT/-124-Luc or pTauT/-124m-Luc (**D**) into HepG2 cells. After transfection, cells were cultured in isotonic medium for 48 h. Results were obtained from six (**B**) or four (**D**) experiments. (**C**, **E**) pFLAG-DN-TonEBP or empty vector was co-transfected with p2xTonE-Luc (**C**) or pTauT/−124-Luc (**E**) into HepG2 cells. Transfected cells were exposed to isotonic or hypertonic media for 24 h. Results were obtained from four experiments. Promoter activity was normalized with luciferase activity of pRL-tk. Results are means ± S.D., $n = 3$–6. $^{*}P < 0.05$, $^{**}P < 0.01$ versus isotonic condition; $^{\#}P < 0.01$ versus empty vector-transfected cells.

cells. Transfection of pFLAG-TonEBP led to significant augmentation of the promoter activity of both p2xTonE-Luc and pTauT/−124-Luc, but not pTauT/−124m-Luc, even in an isotonic medium (Figures 5B and 5D), similar to the previous observations in *hsp70-2* gene regulation [18].

It is well established that the truncated TonEBP, lacking the C-terminus, functions as a DN-TonEBP [10,23]. Therefore we used the expression vector of a truncated protein including 212–562 amino acids of TonEBP, pFLAG-DN-TonEBP, as a DN-TonEBP. In cells transfected with pFLAG-DN-TonEBP, promoter activities of both p2xTonE-Luc and pTauT/−124-Luc were significantly lower in both isotonic and hypertonic conditions compared with cells transfected with the empty vector (Figures 5C and 5E). These results indicated that the TauT promoter activity was regulated through TonEBP in response to hypertonic stress.

Cytoprotective effects of taurine against hypertonicity-induced cell damage

To clarify the biological significance of TauT up-regulation in hypertonic conditions, cells were cultured in hypertonic medium containing 100 mM sucrose with or without 20 μM taurine. As shown in Figures 6(A)–6(C), cell morphology was monitored during incubation. Cells, exposed to hypertonic condition, showed cell shrinkage (Figure 6B). In contrast, this hypertonic-induced cell damage was prevented by supplementation with 20 μM taurine (Figure 6C). Next, to estimate the cell viability, MTS assay was performed (Figure 6D). After culturing under hypertonic condition without taurine for 24 and 48 h, cell viability decreased by 64 and 68 % respectively compared with that of cells cultured in isotonic medium. Treatment of cells with 20 μM taurine significantly recovered cell viability under hypertonic conditions. B-alanine, which is an amino acid analogue of taurine, is mainly taken up by TauT as well as taurine, at 20 μM, also recovered cell viability.

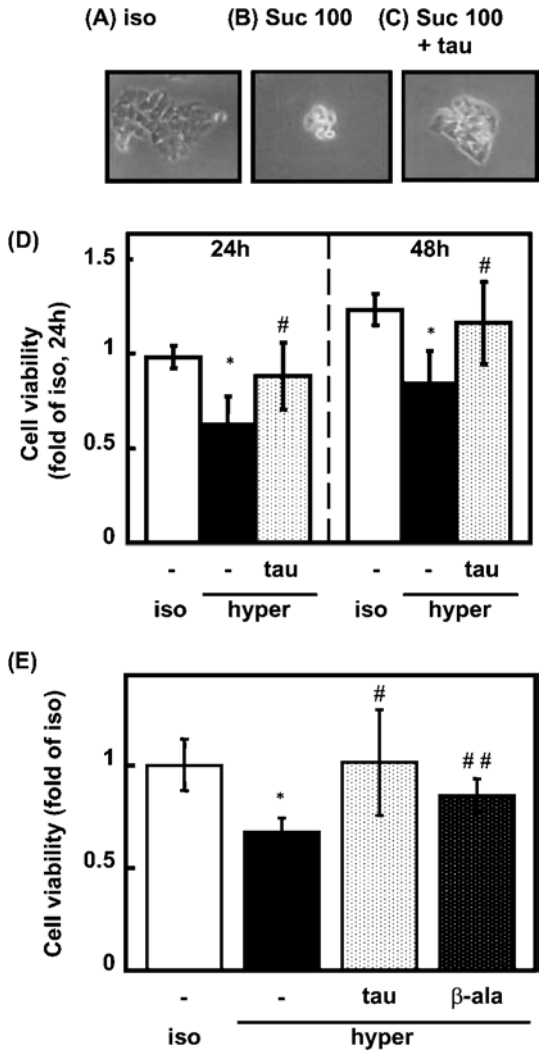

Figure 6 Effect of taurine on the viability of cells exposed to hypertonic stress

Cells were incubated in isotonic medium (**A**) or in 100 mM sucrose-containing hypertonic medium with (**C**) or without (**B**) 20 μM taurine (tau) for 48 h. (**D**, **E**) MTS assay. Cells were cultured in hypertonic medium (Suc 100) with 20 μM taurine for 24 or 48 h (**D**) or cultured with taurine (tau) or β-alanine (β-ala) at 20 μM for 48 h (**E**), and then MTS assay was performed. Results are means $\pm$ S.D., $n = 5$–6. $^{*}P < 0.01$ versus isotonic condition; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus hypertonic condition.

DISCUSSION

In the present study, we identified a TonE consensus motif from −110 to −100 bp of the TauT promoter region. This site is necessary and sufficient for the activation of TauT promoter by hypertonicity. Moreover, we demonstrated that overexpression of TonEBP increased TauT TonE promoter activity under isotonic conditions, whereas co-expression of DN-TonEBP reduced the activity in isotonic and hypertonic conditions. Additionally, taurine prevented HepG2 cells from hypertonicity-induced damage. These findings implied that TauT is up-regulated through the TonE/TonEBP signalling pathway, leading to cytoprotection against hypertonicity. Induction of TauT by hypertonicity has been observed previously in various organs such as kidney [24], brain [25] and liver [26], and also in cultured cell lines such as MDCK cells [27], Caco-2 cells [17] and hepatocytes [26,28] although the mechanisms have not yet been elucidated. Since TonEBP is ubiquitously expressed, our findings provide the possibility that TonEBP regulates cell survival through TauT/taurine signals in a wide range of cell lineages.

In the present study, we identified the TonE site at −110 to −100 bp of a TauT promoter region. We have noticed that a mouse *TauT* gene contains another TonE motif at 6 kb upstream from the transcriptional start site. However, it is unlikely that this distal TonE motif is involved in the regulation of *TauT* gene, because the reporter plasmid containing the distal TonE-like sequence was not activated by hypertonicity (results not shown). Moreover, we confirmed that this TonE-like sequence is not conserved in human and rat *TauT* gene. In contrast, the TonE site, identified in this study, is conserved among rat, mouse and human, supporting the functional significance of this site. Although the TonE sequence of a TauT promoter region was complementary to the consensus sequence, TonEBP overrides the complementary sequences, as demonstrated in urea transporter (UT-A) expression [18].

Although the essential role of TonEBP is not well understood, it is probable that TonEBP plays a central role in protecting mammalian cells from hypertonic stress. Activation of TonEBP by hypertonic stress induces various osmoprotective molecules, including aldose reductase, betaine γ-aminobutyric acid transporter and hsp70-2 (heat-shock protein 70-2) [11,18,29,30]. Moreover, T-cells expressing DN-TonEBP (NFAT5) were observed to reduce viability under hypertonic conditions [10]. In the present study, we demonstrated that a low dose of taurine, taken up only via TauT, a high-affinity taurine transport system [31], suppressed hypertonicity-induced cell damage in HepG2 cells, indicating that

TauT-dependent taurine uptake is a novel cytoprotective mechanism mediated by TonEBP.

Hepatocytes are exposed to hyperosmolality *in vivo* under physiological or pathological conditions, such as nutrient uptake and hypernatraemia [32,33]. Hypertonic stress has been reported to confer susceptibility to heat shock, ischaemia, insulin and CD95 ligand in hepatocytes, which is overcome by taurine [34–37]. These findings supported that the cellular accumulation of taurine via TauT in hepatocytes exerts protective effects against hypertonic stress *in vivo* [35]. To clarify the functional significance of TauT more precisely, hepatic functions should be examined in TauT-null mice.

In summary, *TauT* gene expression is regulated by TonEBP as a protective response to hypertonicity. Activation of the TonEBP/TauT pathway could contribute to prevention of hypertonicity-related tissue damage.

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